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Unbiased label-free quantitative proteomic profiling and enriched proteomic pathways in seminal plasma of adult men before and after varicocelectomy

M. Camargo[1], P. Intasqui Lopes[1], P.T. Del Giudice[1], V.M. Carvalho[2], K.H.M. Cardozo[2], C. Andreoni[1], R. Fraietta[1], and R.P. Bertolla[1,*]

[1]Division of Urology, Department of Surgery, Human Reproduction Section, Sao Paulo Federal University, Sao Paulo Hospital, R Embau, 231, 04039-060, Sao Paulo,SP, Brazil [2]Fleury Group, Sao Paulo, Brazil

*Correspondence address. Fax: +55-11-5573-0014; E-mail: rbertolla@yahoo.com

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STUDY QUESTION: Does the seminal plasma proteomic profile and functional enrichment of gene ontology terms change after microsurgical varicocelectomy? Are there any potential targets for diagnosis or therapeutic intervention in varicocele?

SUMMARY ANSWER: A shift in state from a responsive-to-stress condition before varicocele correction to a responsive-to-environment condition after varicocelectomy was observed in enriched proteomic pathways.

WHAT IS KNOWN ALREADY: Varicocele may lead to many adverse effects, including failure of testicular growth and development, and is associated with decreased semen quality and increased semen oxidative stress. Varicocelectomy is the treatment of choice, and is associated with improved semen quality, but little is known regarding the underlying molecular mechanisms and post-genomic pathways following intervention.

STUDY DESIGN, SIZE, DURATION: A prospective study was carried out including 18 adult men with varicocele. These patients provided one semen sample before they were submitted for bilateral varicocele repair through microsurgical varicocelectomy, and one other semen sample 90 days after the surgery.

PARTICIPANTS/MATERIALS, SETTING, METHODS: An aliquot of each semen sample was used for unbiased proteomics analysis by a label-free quantitative approach (*2D nanoUPLC-ESI-MS^E*). Samples were pooled according to group (normalized to protein content) and run in quadruplicate. These quadruplicate runs provided degrees of freedom in order to compare groups using a non-parametric Mann–Whitney test for quantified proteins.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 316 proteins were quantified or identified, of which 91 were exclusively identified or quantified in one of the groups (53 in the pre- and 38 in the post-varicocelectomy group), and 68 were quantified in both groups and submitted to statistical analysis, of which 5 were overrepresented in the pre-varicocelectomy group ($P < 0.05$). In enriched functional analysis, binding and response to stimulus functions were enriched in a common cluster (present in both groups), nitric oxide metabolism and tetratricopeptide repeat domain-binding functions were enriched in the pre-varicocelectomy group, and response to reactive oxygen species, gluconeogenesis, nicotinamide adenine dinucleotide-binding and protein stabilization were enriched in the post-varicocelectomy.

LIMITATIONS, REASONS FOR CAUTION: Because a shotgun proteomics analysis was chosen in order to generate a list of putative biomarkers, a targeted follow-up study should be performed to confirm these biomarkers.

WIDER IMPLICATIONS OF THE FINDINGS: The proteins found in both groups possess functions usually found in human semen. The enriched function analysis demonstrated a shift back to homeostasis after varicocelectomy, suggesting that varicocele correction promotes return of semen to a physiological state.

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Key words: male infertility / varicocele / varicocelectomy / seminal plasma / proteomics

Introduction

It is estimated that 15% of couples seek clinical help for infertility, and in these a male factor is present in about 50% of cases (Brugh and Lipshultz, 2004). Male infertility is multifactorial, but varicocele is an important and treatable cause (Simpson and Rausch, 2009). While varicocele may be diagnosed in 15% of the adult male population, 40% of men with primary infertility and 80% of men with secondary infertility exhibit varicoceles (Brugh *et al*., 2003). Thus, it has been suggested that varicocele causes a progressive decline on male fertility (Evers and Collins, 2003).

Varicocele is defined as an abnormal dilatation of the efferent veins in the pampiniform plexus (Brugh *et al*., 2003). Adverse effects attributed to this include failure of testicular growth and development, seminal abnormalities, increased scrotal temperature, oxidative stress and Leydig cell dysfunction (WHO, 1992).

Varicoceles have been associated with decreased semen quality (Fariss *et al*., 1981; Sigman and Jarow, 1997; Lung and Larsen, 1998), sperm DNA integrity and mitochondrial activity (Blumer *et al*., 2008, 2012) as well as increased semen oxidative stress (Weese *et al*., 1993; Sharma *et al*., 1999; Blumer *et al*., 2012). However, varicoceles are not always associated with male infertility, since many men with varicoceles of high grade are still able to father children (Nilsson *et al*., 1979).

Current American Society for Reproductive Medicine guidelines recommend treating a varicocele in adult men when a palpable varicocele is present, with at least one abnormal semen parameter, when the couple present with infertility, or when the female counterpart presents a good fertile potential (The Practice Commitee of the American Society for reproductive Medicine, 2008). Treatment of varicoceles is usually achieved by surgical ligation of the dilated veins—varicocelectomy (McClure and Hricak, 1986; The Practice Commitee of the American Society for reproductive Medicine, 2008). A number of methods have been developed for varicocelectomy. A microsurgical subinguinal approach has been shown to produce the lowest recurrence rates (Marmar *et al*., 1985).

Many studies have demonstrated that varicocelectomy is associated with improved semen quality (Schlesinger *et al*., 1994; Madgar *et al*., 1995; Nieschlag *et al*., 1998), sperm DNA integrity and mitochondrial activity (Lacerda *et al*., 2011) and improved outcome in assisted reproduction cycles (Schlesinger *et al*., 1994; Schlegel, 1997; Esteves *et al*., 2010). However, little is known regarding the underlying molecular mechanisms and post-genomic pathways—reflective of an improved testicular environment—present following intervention.

These post-genomic pathways may be studied in sperm or in seminal plasma. Observing the seminal plasma is of special importance because of its impact on the survival of sperm and for successful fertilization (Henault and Killian, 1996). Seminal plasma is the liquid component of semen composed of lipids, proteins, sugars and metabolites that interact to provide sperm with a safe path in the female reproductive tract. The average concentration of proteins in human seminal plasma ranges from 35 to 55 g/l, which makes it an easy, rich and easily accessible source for protein identification (Pilch and Mann, 2006). The protein content of seminal plasma has a fundamental role in reproduction, because it will potentially determine the fertilizing capacity of sperm (Amann, 1989). For a proteomics approach, both targeted and untargeted techniques may be applied. While targeted proteomics aims at quantifying proteins participating in suspected pathways, untargeted proteomics allows a complete screening of proteins in a sample with an unbiased approach. Thus, techniques which identify and quantify the largest amount of proteins are utilized in order to reveal putative pathways (Wilkins *et al*., 1996). Bioinformatic filtering of this large amount of information is extremely important in order to filter out noise, and the use of functional enrichment of gene ontology (GO) terms, and thence proteins that generate these hits, is a helpful means of generating candidate biomarkers (Quackenbush, 2007).

For generation of a large amount of proteomics information, mass spectrometry (MS)-based methods, coupled to liquid chromatography preseparation are widely and routinely used. MS-based proteomics as a discovery platform has revolutionized our current understanding of molecular pathways, because the technology utilized is able to identify, quantify and elucidate the sequence of a variety of isolated molecules in the ionized form in a selective, highly sensitive and reliable manner (Gaviraghi *et al*., 2010).

An approach used in MS-based proteomics is label-free quantification by MS^E data-independent acquisition. In this method, alternating scans of low and elevated collision energy throughout chromatographic analysis allows the multiplexed acquisition of peptide fragmentation regardless of their intensity (Silva *et al*., 2005). The association of precursor and product ions is performed by data modeling taking into account 14 physicochemical features to maximize peptide identification confidence and subsequent protein identification. The absolute quantification is achieved by the average signal intensity of the three most intense peptides produced by digestion with trypsin (Silva *et al*., 2006). Regarding proteomics, this allows quantification of individual proteins while still maintaining an unbiased approach (Plumb *et al*., 2006).

Conventional semen analysis as an evaluation of male infertility has been shown to be flawed and of little predictive value (Guzick *et al*., 2001). Novel techniques, such as analysis of sperm DNA integrity and mitochondrial activity have shed important light into understanding male infertility. In adolescents, for example, it has been demonstrated that the presence of a varicocele leads to altered sperm DNA integrity even when semen analysis is unaffected (Bertolla *et al*., 2006). In adults, varicocele and its effects are best observed by sperm function, not by conventional semen analysis (Blumer *et al*., 2008). However, although our knowledge of the detrimental effects of varicocele has increased, the molecular pathways are still quite unclear. The paradigm-shifting approach of using post-genomic pathways thus promises to explain in a more precise manner the

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'How' in varicocele-derived male infertility, and in its treatment. Thus, the aim of this study was to evaluate the proteomic profile of patients before and after microsurgical varicocelectomy, and to compare these profiles through the study of enriched GO functions, in order to generate proteomic pathways associated with each condition.

Materials and Methods

Patients

Institutional Review Board approval was obtained from the Sao Paulo Federal University Research Ethics Committee. A prospective study was carried out including 18 adult men with varicocele. Inclusion criteria were adult men between 20 and 40 years of age, with surgical indication, referred to the Division of Urology of the Sao Paulo Federal University.

Clinical parameters

Varicocele was diagnosed by clinical analysis, including scrotal palpation in a temperature-controlled room (>23°C) with adequate illumination, and varicocele was graded according to Dubin and Amelar (1977). The criteria used were:

(i) Varicocele grade I: dilatation of spermatic cord palpable only with Valsalva maneuver;
(ii) Varicocele grade II: dilatation of spermatic cord easily palpable, with the patient standing, demonstrating marked venous dilatation during Valsalva maneuver;
(iii) Varicocele grade III: massive dilatation of spermatic cord easily visualized with patient standing and intensified ectasia during Valsalva maneuver.

Exclusion criteria

Patients referring fever in the 90-day period prior to semen analysis, with evidence of urogenital infection, and patients with a history of cancer or endocrinopathies (and/or their potentially gonadotoxic treatments) were excluded from the study.

Thus, patients were grouped as before (pre) and after (post) microsurgical varicocelectomy in order to verify the effect of varicocelectomy itself (intervention study).

Intervention

Patients provided two semen samples, one before microsurgical varicocelectomy and one 90 days after. Semen samples were collected by masturbation after 2–5 days of ejaculatory abstinence. After liquefaction, the samples were analyzed according to the WHO (World Health Organization, 1999), and sperm morphology according to Kruger's strict criteria (1986).

All patients included in the study were submitted to bilateral varicocele repair using subinguinal microsurgical varicocelectomy according to Marmar *et al*. (1985).

Proteomic analysis of seminal plasma

Protein quantification

Seminal plasma samples were thawed and centrifuged at 16 100*g* for 30 min at 4°C to remove cellular debris and the supernatant was used. Following centrifugation, total protein concentration from each sample was evaluated using the bicinchoninic acid (BCA) protein assay (modified Lowry method—Sigma, St Louis, MO, USA; Smith *et al*., 1985). Each sample was measured in triplicate, and a standard curve was generated in duplicate. Samples with coefficients of variation over 10% were re-measured.

The samples from different patients were pooled according to the group (pre × post-varicocelectomy) and normalized according to protein concentration, in which each sample contributes with the same amount of total protein. Eight pools were formed: (i) four including the seminal plasma from all the patients before varicocelectomy surgery and (ii) four including the seminal plasma from all the patients after varicocelectomy surgery. The four pools pre- and the four pools post-varicocelectomy comprised the same patients at two different times. These pools were then quantified in triplicate using the BCA protein assay.

Protein digestion

For each study group, four technical replicates were separately prepared. Initially, a volume corresponding to 50 μg total protein was diluted in Milli-Q water to a volume of 30 μl. The protein (fluid extract) samples were denatured with 0.2% (w:v) RapiGest SF protein surfactant for 15 min at 80°C. These were then reduced in the presence of 2.5 μl of 100 mM dithiothreitol at 60°C for 30 min, alkylated with 2.5 μl of 300 mM iodoacetamide at room temperature, and enzymatically digested at 37°C overnight with trypsin (Sequencing Grade Modified Trypsin, Promega, WI, USA) at a 1:100 (w/w) enzyme:protein ratio. Then, 10 μl of 5% trifluoroacetic acid was added to the digestion mixture in order to hydrolyze the RapiGest and the samples were incubated at 37°C for 90 min. The tryptic peptide solution was then centrifuged at 16 000*g* for 30 min at 6°C and the pH of the supernatant was adjusted to 9.8 by the addition of 1 M NH_4OH. Finally, 5 μl of the internal standard (yeast alcohol dehydrogenase—P00330 at 250 fmol/μl) was added to the resulting solution.

Liquid chromatography/MS^E analysis

Ten microliters of each digested sample (corresponding to 5 μg of total protein digests) were injected into a 2D Technology nanoACQUITY ultra-pressure liquid nanochromatography system. This approach combines separation using pH 10 in the first and pH 2.6 in the second separation dimension using reverse phase columns (Gilar *et al*., 2005). First dimension separation was achieved in an XBridge BEH130 C18 5 μm 300 μm × 50 mm column. Elution was performed using 10 different binary gradients with 20 mM pH 10 ammonium formate in acetonitrile at 2 μl/min. The proportion of acetonitrile from the first to the 10th fraction ranged from 7.4 to 65% of acetonitrile. Eluted peptides from the first dimension column were trapped in a symmetry C18 5 μm 180 μm × 20 mm column and on-line diluted with 0.1% formic acid with 0.1% acetonitrile at a flow rate of 20 μl/min. Second dimension separation was performed in a HSS T3 1.8 μm 75 μm × 100 mm using a binary gradient from 7 to 40% of acetonitrile in 0.1% formic acid during 54 min at a flow rate of 400 nl/min.

Mass spectrometric acquisition was achieved in a Synapt MS Q-TOF mass spectrometer equipped with a nanolockspray source in the positive ion mode (Waters, Manchester, UK). For all measurements, the mass spectrometer was operated in the 'V' mode with a typical resolving power of at least 12 500. The mass spectrometer was calibrated with 200 fmol/mi Human [Glu^1]-fibrinopeptide B (GFP) delivered through the reference sprayer of the NanoLockSpray source. The doubly charged ion ($[M + 2H]^{2+}$) was used for initial single-point calibration and MS/MS fragment ions of GFP were used to obtain the final instrument calibration. The lock-mass correction was achieved sampling GFP ions every 30 s. Data-independent scanning (MS^E) experiments were performed by switching between low (3 eV) and elevated collision energies (15–50 eV) applied to the trap 'T-wave' cell filled with argon. Scan times of 0.8 s were used for low- and high-energy scans from *m/z* 50 to 2000. The radio frequency offset was adjusted such that the liquid chromatography (LC)/MS data were effectively acquired from *m/z* 300 to 2000,

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which ensured that any masses observed in the LC/MS^E data of less than *m/z* 300 were known to arise from dissociations in the collision cell.

Spectra processing and database searching were performed with ProteinLynx Global Server v.2.5 (PLGS). A UniProtKB/Swiss-Prot Complete Proteome database Release 2012_02 was used and the search conditions were based on taxonomy (*H. sapiens*). The randomized database was appended to the original database to assess the false discovery rate, set up to 4%. Maximum missed cleavages by trypsin allowed were up to 1, fixed modification by carbamidomethylation (cistheine) and variable modifications by acetyl N-terminal and oxidation (methionine) were considered. Label-free quantification was obtained by the PLGS processing and lists of identified and/or quantified proteins in each condition (pre- and post-varicocelectomy).

Statistical analysis

All statistical analyses were conducted in SPSS. For semen analysis, data were initially submitted to a Kolmogorov–Smirnov normality test to verify whether a parametric test could be applied. If so, pre- and post-varicocelectomy samples were compared using a Student's *t*-test for paired samples. If not, then this analysis was carried out using a Wilcoxon signed-ranks test. An alpha of 5% was utilized.

For proteomics analysis, only proteins present in at least two of the four replicates were included in this study. When proteins were not quantified, they were assigned as 'identified' and included in exclusive expression analysis but not in statistical analysis. Furthermore, if a protein was quantified in one group but only identified in the other, no statistical inference could be made, and thus it was also excluded from the differential analysis.

For the statistical analysis of quantitative data, initially a descriptive analysis was performed for calculating the mean of the four replicates and the fold-change (ratio between the mean of each group). Positive fold-change values represent a higher protein expression in the post-varicocelectomy group, while negative values show a higher expression in the pre-varicocelectomy group. Because in a small sample size ($n = 4$ in each group) normality assumptions may not be tested for, groups were compared using a Mann–Whitney test ($P < 0.05$).

Functional enrichment analysis

A list of the set of proteins expressed exclusively or differentially in each group ($P < 0.05$) was generated, and these data were initially submitted to biological network analysis using Cytoscape 2.8.2 (Smoot *et al.*, 2011). Protein–protein interaction searches were performed using the Bisogenet plugin (Martin *et al.*, 2010), which in turn searches the human protein interaction databases IntAct (Kerrien *et al.*, 2007), BIND (Alfarano *et al.*, 2005), DIP (Salwinski *et al.*, 2004), HPRD (Mishra *et al.*, 2006), BioGRID (Stark *et al.*, 2006) and MINT (Zanzoni *et al.*, 2002). Because interaction searches are able to generate clusters of interacting proteins, these clusters may be searched for statistically enhanced functions. Thus, all clusters were submitted to functional enrichment analysis with the ClueGO plugin (Bindea *et al.*, 2009). The ClueGO plugin calculates statistically overrepresented GO terms (The Gene Ontology Consortium, 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000) in each group, and builds an enrichment pathway. GO results are shown as functions statistically over-represented ($P < 0.05$) in three major categories: (i) cellular component, (ii) molecular function and (iii) biological process. KEGG pathways are overlaid to these GO terms to build an enrichment map for (i) protein clusters present in both groups, (ii) proteins in the pre-varicocelectomy group and (iii) proteins in the post-varicocelectomy group.

Results

Clinical data regarding semen analysis in all the patients are presented in Table I. Only rapid progressive motility (Grade a) was significantly

Table I Clinical and semen analysis data of adult men before (pre) and 90 days after (post) microsurgical subinguinal varicocelectomy.

	Pre ($n = 18$)	Post ($n = 18$)	*P*
Volume (ml)			
Mean ± SD	3.3 ± 1.31	3.8 ± 1.79	0.051
95% CI	2.7–4.0	2.9–4.7	
Motility grade a* (%)			
Median ± IR	0 ± 2	3 ± 8	0.04995$^+$
25th–75th percentile	0–2.0	0–8	
Motility grade b* (%)			
Mean ± SD	42.6 ± 22.80	48.2 ± 15.44	0.22
95% CI	31.3–54.0	40.5–55.9	
Progressive motility (%, a + b)			
Mean ± SD	44.3 ± 24.00	52.7 ± 17.74	0.06
95% CI	32.3–56.2	43.8–61.5	
Motilitygrade c* (%)			
Mean ± SD	5.5 ± 3.47	5.12.92	0.61
95% CI	3.8–7.2	3.6–6.5	
Motility grade d* (%)			
Mean ± SD	50.2 ± 22.98	42.3 ± 16.12	0.06
95% CI	38.8–61.7	34.3–50.3	
Concentration ($\times 10^6$/ml)			
Median	5.1	13.9	0.68
25th–75th percentile	1.3–26.0	2.1–25.4	
Morphology (% normal)[a]			
Mean ± SD	4.2 ± 2.99	4.4 ± 2.20	0.76
95% CI	2.5–5.9	3.2–5.6	
Round cells ($\times 10^6$/ml)			
Mean ± SD	2.2 ± 2.50	2.0 ± 2.34	0.81
95% CI	1.0–3.5	0.72–3.0	
Neutrophils ($\times 10^6$/ml)			
Median ± IR	0.2	0.1	0.46
25th–75th percentile	0–1.0	0–0.4	

Groups were compared using a Student's *t*-test for paired samples (Motility grade 'a', sperm concentration, and neutrophil count were compared using Wilcoxon signed-ranks test). An alpha of 5% was adopted.
SD, standard deviation; 95% CI, 95% confidence interval of the mean; IR, interquartile range.
*Grade a: rapid progressive; Grade b: progressive; Grade c: non-progressive; Grade d: static.
$^+$Statistically significant difference ($P < 0.05$).
[a]For morphology, four samples were not analyzed in the pre-varicocelectomy group, and three in the post-varicocelectomy group due to low sperm count. Thus, for morphology, $n = 14$ and 15 in these two groups, respectively.

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different between pre- and post-varicocelectomy samples. Age (mean; standard deviation) was 30.6; 4.7 years. Of the 18 men included in this study, 10 presented with bilateral varicocele grade II left, grade I right, 2 presented with bilateral varicocele grade II left, grade II right, 2 presented with bilateral varicocele grade II on both sides, 1 presented with bilateral varicocele grade III in both sides, 1 presented with varicocele grade III left, grade I right, 1 presented unilateral varicocele grade III left and 1 presented with unilateral varicocele grade II left.

In this study, 316 proteins were identified before and after surgery in seminal plasma of varicocele patients. A full list of these proteins may be observed in Supplementary data, Table SI. One hundred and twenty-one were identified in both groups, but were below the quantification threshold; these were then excluded from the statistical analysis, as were 36 proteins that could be quantified in one group but only identified in the other. It is important to note that these were included in the interactome and functional enrichment analysis because, even though they were not proved to be statistically significantly different between groups, they build up the interaction networks and thus should not be excluded. A total of 159 proteins were used for comparative analysis, of which 91 were exclusive to either one of the two groups, and 68 were quantified in both groups and submitted to statistical analysis.

Of the 91 exclusively expressed proteins, 30 were quantified, 19 in the pre-varicocelectomy group and 11 in the post-varicocelectomy group, and 61 were identified, 34 in the pre-varicocelectomy group and 27 in the post-varicocelectomy group (Table II).

The 68 quantified proteins are presented in Table III. Of these, five presented a significant difference in expression between groups ($P < 0.05$). For these, fold changes were calculated using the pre-varicocelectomy group as a reference (negative values indicate a decrease in expression after varicocelectomy).

An overview of the protein–protein interaction network from this study is presented in Fig. 1. Yellow nodes represent the proteins that were not different between groups, red nodes represent proteins exclusively or overexpressed in the pre-varicocelectomy group and green nodes represent proteins exclusively observed in the post-varicocelectomy group. Functional enrichment of interactome clusters are presented in Fig. 2. Binding and response to stimulus functions were enriched in the common cluster, which presented enriched nitric oxide (NO) metabolism and tetratricopeptide repeat (TPR) domain-binding before varicocelectomy, and response to reactive oxygen species, gluconeogenesis, nicotinamide adenine dinucleotide (NAD)-binding and protein stabilization after varicocelectomy.

Discussion

To investigate the consequences of microsurgical varicocelectomy for the seminal plasma protein profile of patients with varicocele, a shotgun proteomics (protein *fingerprinting*) study of these patients was performed. A label-free technique which allows quantification of proteins was used. Ultra-performance liquid chromatography equipment (McKenna *et al*., 2007) was coupled to a mass spectrometer operating in alternating low/high collision energies to generate quasi-simultaneous MS/MS spectra in a data-independent manner (Silva *et al*., 2006). This maximized the certainty of quantification (due to improved lossless total ion chromatogram) while maintaining a shotgun approach (Plumb *et al*., 2006). It is especially important to note that selecting this approach allows an unbiased approach toward proteomics investigation, and the non-assumption of putative proteomic pathways allows the generation of hypotheses-generating results while still allowing for a Cartesian approach (Silva *et al*., 2006).

Current proteomic online databases assign a number of functions (GO terms) to proteins, and any individual protein may present a number of GO terms associated with it. However, it is especially important to note that these proteins will most likely (statistically) present functions when interacting with other proteins in a protein–protein interaction network. This principle—functional enrichment—allows statistical inference in protein interaction networks and is based on the emerging knowledge that genes (and gene products) do not participate individually in determining phenotype, but rather interact in scale-free format networks to develop specified functions (The Gene Ontology Consortium, 2000). Finally, the fact that these networks are scale-free is important because it clearly demonstrates that few proteins interact with many proteins, while many proteins interact with only a few (Kunin *et al*., 2004). This concept dates back to the concept of lethal genes, the disruption of which leads to important phenotypic alterations, while disruption of non-lethal genes generally leads to milder alterations in phenotype (Park *et al*., 2008). In this study, all the patients had mature sperm in the ejaculate and must have had a complete spermatogenic cycle in at least some seminiferous tubules, and any alteration in their fertility status is most likely to be associated with differences in non-lethal gene products (proteins with few interactions), while more central proteins are unaltered.

A network with all 316 proteins found in this analysis was constructed, which allowed observation of the proteome shift between the pre- and the post-varicocelectomy samples. It is of interest to observe that a representative portion of the proteome is conserved, indicating preserved functions in the varicocele samples.

Conserved proteins

These conserved proteins are secreted by the prostate and seminal vesicles (Purvis *et al*., 1986; Milardi *et al*., 2012), such as kallikrein (P20151, P07288), cathepsin (P07858, P07339), cystatin (P01034), fibronectin (P02751), semenogelin (P04279, Q02383), actin (P62736, P60709, P68032, P63261), polyubiquitin-C (P0CG48), peroxiredoxin (P06830) and proteins with immune functions (P01834, P01857, P01861). Functional enrichment analysis demonstrates that binding function and response to stimulus are enriched in this group of proteins. This suggests that this group of proteins present in seminal plasma of mammals is involved in the steps that precede fertilization, such as the regulation of capacitation, modulation of uterine immune response and transport of sperm in the female genital tract, as well as gamete interaction and fusion (Töpfer-Pertensen *et al*., 2005; Milardi *et al*., 2012).

Polyubiquitin-C is the protein with the largest number of protein–protein interactions in this cluster (Fig. 2). It binds to proteins common to both groups and with proteins exclusively or differentially expressed in pre- and post-varicocelectomy groups. Its role is to mark proteins for degradation by lysosomes and proteasomes (Hochstrasser, 1996). Sutovsky *et al*. (2001) proposed the existence of an operating system mediated by ubiquitin in the epididymis of mammals dedicated to recognize and mark defects in sperm (Sutovsky *et al*., 2001). They

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Table II **Proteins in pooled seminal plasma samples of adult men exclusively expressed either before (pre) or 90 days after (post) microsurgical subinguinal varicocelectomy.**

UniProt AC	Protein description	Mean (fmol/column)	
		Pre	Post
P62258	14-3-3 Protein epsilon YWHAE 1 1	7.2	–
P27348	14-3-3 Protein theta YWHAQ 1 1	9	–
A8MTF8	Family with sequence similarity 3_ member B	Identified	–
A8MXQ4	L-lactate dehydrogenase	Identified	–
Q06481	Amyloid-like protein2	Identified	–
Q9UHI8	A disintegrin and metalloproteinase with thrombospondin motifs 1	13	–
B1APH0	Basonuclin 2	Identified	–
P80723	Brain acid soluble protein 1	37.8	–
Q6ZN30	Zinc finger protein basonuclin-	145.5	–
C9IZG4	CutA divalent cation tolerance homolog *Escherichia coli*	39.5	–
C9J5G4	Follistatin-like 1 Fragment	Identified	–
C9JZR7	Actin_ beta Fragment	Identified	–
O60888	Isoform A of Protein CutA	Identified	–
D6REQ6	Ribonuclease T2	Identified	–
E5RIZ5	Peptidylprolylisomerase A cyclophilin A	Identified	–
E9PCB3	Uncharacterized protein CTSB 4	Identified	–
E9PHZ5	Cathepsin B Fragment	Identified	–
E9PJ67	Cathepsin B Fragment	Identified	–
E9PKD0	Cathepsin B Fragment	Identified	–
E9PKQ7	Cathepsin B Fragment	Identified	–
E9PLY3	Cathepsin B Fragment	Identified	–
E9PNL5	Cathepsin B Fragment	Identified	–
E9PQM1	Cathepsin B Fragment	Identified	–
E9PQS3	Amyloid beta A4 precursor-like protein 2 Fragment	Identified	–
E9PR54	Cathepsin B Fragment	Identified	–
E9PSG5	Cathepsin B Fragment	Identified	–
F5GXY2	Lactate dehydrogenase A Fragment	Identified	–
F5GYU2	Lactate dehydrogenase A Fragment	Identified	–
F5GZY0	Amyloid beta A4 precursor-like protein 2	Identified	–
F5H586	Basonuclin 2	Identified	–
F5H8G9	Basonuclin 2	Identified	–
F8W7I2	Prostaglandin D2 synthase 21kDa brain	Identified	–
F8W819	L-lactate dehydrogenase	Identified	–
P58499	Protein FAM3B	19.2	–
P09211	Glutathione S-transferase P	23.5	–
H0Y4U4	Progestagen-associated endometrial protein Fragment	349.5	–
H0Y6W5	Basonuclin 2 Fragment	Identified	–
H0YA83	Hexosaminidase B beta polypeptide Fragment	Identified	–
H0YAM0	Carboxypeptidase E Fragment	Identified	–
P07900	Heat shock protein HSP 90-alpha	20.1	–
P08238	Heat-shock protein HSP 90-beta	14.4	–
B9A064	Immunoglobulin lambda-like polypeptide 5	10.2	–
P0CG05	Ig lambda-1 chain C regions	34.3	–
P00338	L-lactate dehydrogenase A chain	16	–
Q04760	Lactoylglutathione lyase	13.3	–

Continued

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Table II *Continued*

UniProt AC	Protein description	Mean (fmol/column)	
		Pre	Post
Q02818	Nucleobindin-1	36.6	–
Q6S8J3	POTE ankyrin domain family member E	Identified	–
P0CG38	POTE ankyrin domain family member I	Identified	–
P41222	Prostaglandin-H2 D-isomerase	Identified	–
Q86U12	Full-length cDNA clone CS0CAP007YF18 of Thymus of Homo sapiens	Identified	–
Q9NRW1	Ras-related protein Rab-6B	34.3	–
P34096	Ribonuclease 4	24.2	–
Q6PCB0	von Willebrand factor A domain-containing protein 1	22.2	–
A6NNI4	CD9 molecule	–	Identified
P04083	Annexin A1	–	9
B4DPP0	CD9 molecule	–	Identified
B4DUN2	Malate dehydrogenase	–	Identified
B5MC34	Gamma-glutamyltransferase 1	–	Identified
B5MC36	Gamma-glutamyltransferase 1	–	Identified
B5MCK8	Gamma-glutamyltransferase 2	–	Identified
B7WND7	Uncharacterized protein Homo sapiens GGTLC2 4 1	–	Identified
B9A041	Malate dehydrogenase 1_ NAD soluble	–	Identified
C9IYX7	Membrane metallo-endopeptidase Fragment	–	Identified
C9J9X7	Membrane metallo-endopeptidase Fragment	–	Identified
C9JDZ3	Membrane metallo-endopeptidase Fragment	–	Identified
C9JR96	Membrane metallo-endopeptidase Fragment	–	Identified
P21926	CD9 antigen	–	Identified
E5RJI0	Lipoprotein lipase Fragment	–	Identified
E7EPW1	Uncharacterized protein SEMG1	–	Identified
E7ET60	Ribonuclease T2	–	Identified
E7ET76	Gamma-glutamyltransferase 1 Fragment	–	Identified
E7ETR7	Gamma-glutamyltransferase 1 Fragment	–	Identified
E7EUT4	Glyceraldehyde-3-phosphate dehydrogenase	–	Identified
E7EUT5	Glyceraldehyde-3-phosphate dehydrogenase	–	Identified
F5H098	Malate dehydrogenase	–	11.7
F8W825	Nucleolar protein 4	–	Identified
P04406	Glyceraldehyde-3-phosphate dehydrogenase	–	21
G8JLH6	CD9 molecule Fragment	–	Identified
P19440	Gamma-glutamyltranspeptidase 1	–	17.2
P36268	Gamma-glutamyltranspeptidase2	–	6.2
A6NGU5	Putative gamma-glutamyltranspeptidase3	–	Identified
Q14390	Gamma-glutamyltransferase light chain 2	–	Identified
P31025	Lipocalin-1	–	4.8
P40925	Malate dehydrogenase_cytoplasmic	–	Identified
P08473	Neprilysin	–	8.4
Q99497	Protein DJ-1	–	11.8
Q5TCT1	Ribonuclease T2	–	Identified
P20337	Ras-related protein Rab-3B	–	17.3
Q96E17	Ras-related protein Rab-3C	–	Identified
P06702	Protein S100-A9	–	40.3
P00441	Superoxide dismutase [Cu-Zn]	–	38.2

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Table III Differentially expressed proteins quantified in pooled seminal plasma samples of adult men before (pre) and 90 days after (post) microsurgical subinguinal varicocelectomy.

Uniprot AC	Protein description	Mean (fmol/column)		Fold-change	P
		Pre	Post		
P08758	Annexin A5	31.6	10.6	−2.99	0.02
P61769	Beta-2-microglobulin	217.5	173.7		0.51
P49913	Cathelicidin antimicrobial peptide	65.6	65.5		0.77
P07339	Cathepsin D	49.7	48		0.48
Q8N6Q3	CD177 antigen	32.3	28.5		0.48
P10909	Clusterin	2526.1	1536.5	−1.64	0.02
P01037	Cystatin-SN	40.8	4		0.08
D6REA1	Homolog_ endoplasmic reticulum	17.1	16.3		0.56
E7ERT3	Lactotransferrin	561	452.5	−1.24	0.05
E7ESU5	Albumin	1308.1	650.5		0.25
E9PRZ4	Chromosome 11 open reading frame 85	551.5	254		0.48
Q16610	Extracellular matrix protein 1	90.7	83.5		0.38
P06733	Alpha-enolase	24.7	16.5		0.06
Q12841	Follistatin-related protein 1	84.5	53.3		0.77
G3XAE3	Kallikrein 3_ Prostate specific antigen_	2195.4	1617.6		1.00
P02790	Hemopexin	41	26.4		0.29
P18065	Insulin-like growth factor-binding protein 2	16.7	14.8		0.83
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	21.7	12.8		0.25
P01876	Ig alpha-1 chain C region	49.2	33.3		0.25
P01877	Ig alpha-2 chain C region	11.9	7		0.51
P01834	Ig kappa chain C region	146.6	91.3		0.25
Q9UBX7	Kallikrein-11	28.9	91.3		0.35
P20151	Kallikrein-2	154.8	38.3		0.51
P08118	Beta-microseminoprotein	500.4	273.6		0.38
P80188	Neutrophil gelatinase-associated lipocalin	33.1	25.4		1.00
P01833	Polymeric immunoglobulin receptor	29	18.7		0.56
P23284	Peptidyl-prolyl cis-trans isomerase B	45.3	38.1		0.38
P32119	Peroxiredoxin-2	20.4	11.2		0.51
Q5H9A7	TIMP metallopeptidase inhibitor	100.5	75.1		1.00
O00391	Sulfhydryl oxidase 1	49.6	29.2		0.29
P07602	Proactivator polypeptide	151.6	140		0.35
P04279	Semenogelin-1	3661.9	2724.9		0.38
Q02383	Semenogelin-2	5561.9	2846		0.08
P08294	Extracellular superoxide dismutase [Cu−Zn]	20.4	13.2		0.29
P49221	Protein−glutamine gamma-glutamyltransferase 4	72.3	42.3	−1.69	0.02
P01033	Metalloproteinase inhibitor 1	84.4	12.9		0.56
P02787	Serotransferrin	167.6	162.4		0.25
Q14508	WAP four-disulfide core domain protein 2	391.5	106.4		0.08
P25311	Zinc-alpha-2-glycoprotein	1436.8	896.6	−1.60	0.04
A6NBZ8	Uncharacterized protein ALB 4 2	802.3	984.7		0.83
P15144	Aminopeptidase N	29.5	60.4		0.56
B4DVE1	Lectin_ galactoside-binding_ soluble_ 3	3.58	23.2		0.83
C9JKZ3	Transmembrane protease_ serine 2 Fragment	11	24.7		0.12
P16870	Carboxypeptidase E	113.6	181.3		0.38
P54107	Cysteine-rich secretory protein 1	148.6	224.4		1.00

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Table III *Continued*

Uniprot AC	Protein description	Mean (fmol/column)		Fold-change	*P*
		Pre	Post		
P01034	Cystatin-C	225	252.0		1.00
P01036	Cystatin-S	290.1	518.5		0.25
E9PJC5	Mucin 6_ oligomericmucusgel-forming Fragment	117.7	143.1		0.77
F5H0N0	Actin_ gamma 1	20.8	29.6		0.56
F8W7G7	Fibronectin 1	175.8	261.2		0.56
P02751	Isoforms of Fibronectin	387	485.2		1.00
H0Y7Z1	Fibronectin 1 Fragment	268	313.2		0.77
P01857	Ig gamma-1 chain C region	132.5	133.4		0.56
P01859	Ig gamma-2 chain C region	53.1	79.9		0.25
P01860	Ig gamma-3 chain C region	1.6	12.2		0.12
P05154	Plasma serine protease inhibitor	34.2	42.7		0.56
Q08380	Galectin-3-binding protein	225.7	233.4		1.00
P06858	Lipoprotein lipase	22.1	36.8		1.00
P80303	Nucleobindin-2	15.1	64		0.06
P12273	Prolactin-inducible protein	459.6	2764.7		0.15
P53H76	Phospholipase A1 member A	18.6	24.4		1.00
P15309	Prostatic acid phosphatase	1180.5	1605		0.25
Q06830	Peroxiredoxin-1	9.7	9.7		0.64
P62979	Ubiquitin-40S ribosomal protein S27a	23.1	128.5		0.12
P03973	Antileukoproteinase	215.6	219.3		0.56
P49223	Kunitz-type protease inhibitor 3	43.8	53.7		0.51
Q08629	Testican-1 SPOCK1 1 1	36.3	43.6		0.48
P0CG48	Polyubiquitin-C	151.3	174.3		0.56

Positive fold-change values indicate overexpression in the post-varicocelectomy group, while negative values indicate overexpression in the pre-varicocelectomy group. Statistical analysis: Mann–Whitney test ($n = 4$).

observed an increase in the percentage of ubiquitinated sperm along the way from the testis to the epididymides, which decreases after reaching the epididymal tail. It was also observed that the semen ubiquitination is associated with a poor quality of semen parameters (Sutovsky *et al*., 2004); however, Muratori *et al*. (2005) found that ubiquitination of sperm may be related to quality of semen parameters (Muratori *et al*., 2005). The fact that this study found an association of this protein with proteins found in the groups before and after varicocelectomy corroborate these studies, showing that the role of polyubiquitination is conserved, not sensitive to adverse or favorable conditions.

Several actin subtypes were identified in both groups (P62736, P60709, P68032, P63261), being major proteins of the cytoskeleton and participating in many functions such as cell motility, movement of vesicles and organelles, cell signaling, and cell shape maintenance and stabilization (Machesky and Install, 1999). These proteins may have been observed as residual proteins from degrade sperm and other cells in the male reproductive tract. While these are a possible contamination of the seminal plasma sample, it is important to note that they do constitute the proteome of seminal plasma in ejaculated samples, and thus their presence should not be disregarded.

Pre-varicocelectomy proteins

The annexin 5 (P08758), lactate dehydrogenase (P00338), clusterin (P10909), 3.3.14 epsilon (P62258), 3.3.14 theta (P27348), heat-shock protein 90-beta (P08238) and heat-shock protein 90-alpha (P07900) proteins are present in the pre-varicocelectomy group and they relate and participate in functions relating to metabolism and regulation of NO, and of TPR domain-binding, as shown in Fig. 2.

Clusterin (P10909) has key roles inside and outside the cell (Martínez-Heredia *et al*., 2008). It is related to preservation of the damage of oxidative reactions (Reyes-Moreno *et al*., 2002), protein precipitation (Ibrahim *et al*., 2000), agglutination of abnormal spermatozoa (O'Bryan *et al*., 1990, 1994), control of sperm lyses induced by complement proteins (Jenne and Tschopp, 1989), and it can also be considered a biomarker for fertility in stallions and men (Ibrahim *et al*., 2000; Novak *et al*., 2010; Milardi *et al*., 2012), which could explain its link with polyubiquitin protein (P0CG48) and its quantitative increase ($P < 0.05$) in the pre-varicocelectomy group as a control mechanism.

The heat-shock proteins are expressed in Sertoli cell, Leydig cells, spermatocytes, spermatids and spermatogonia (Ogi *et al*., 1999). These proteins re-establish the homeostatic mechanism and

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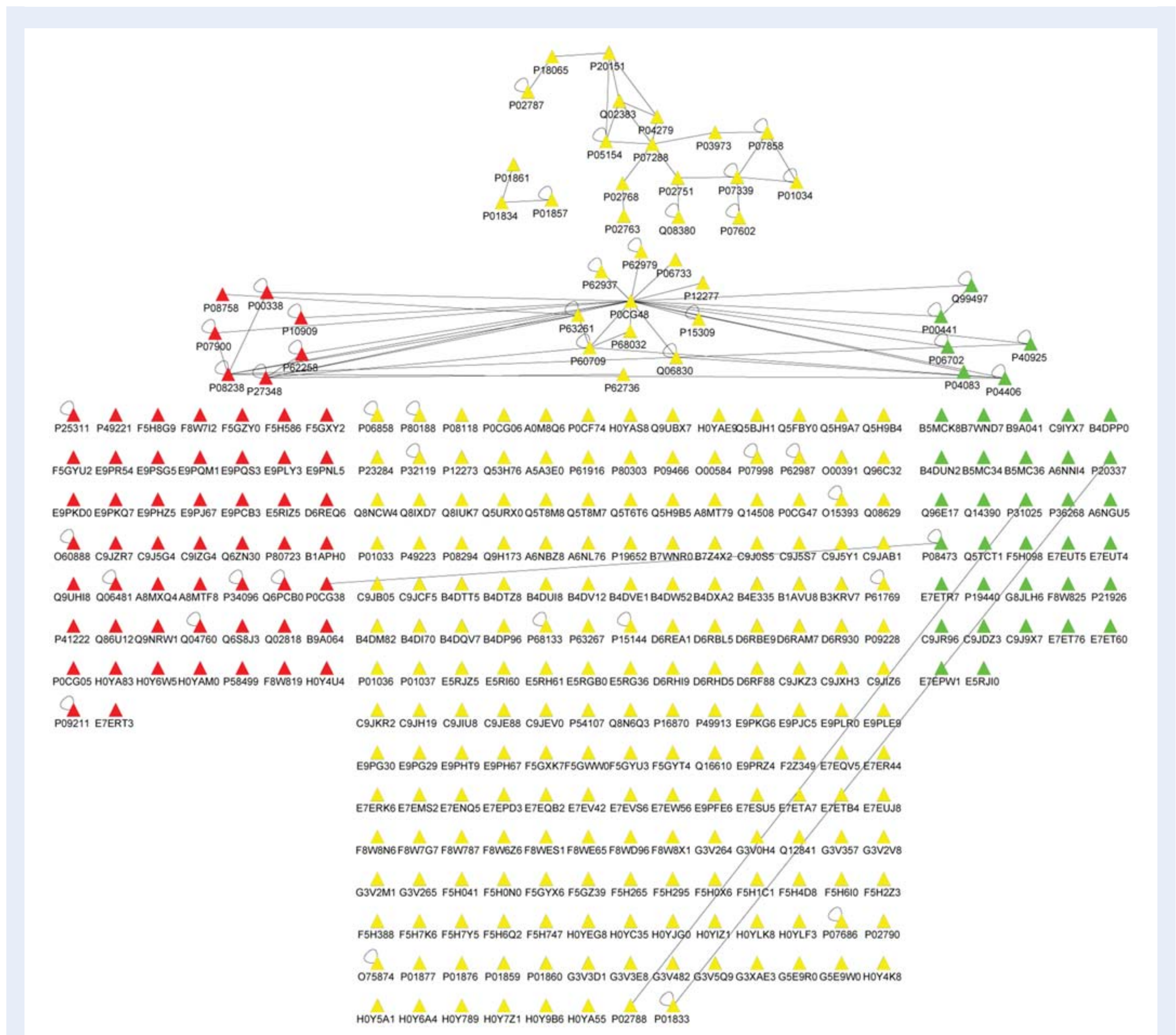

Figure 1 Interactome of the identified or quantified proteins in pooled seminal plasma samples of adult men before (pre) and 90 days after (post) microsurgical subinguinal varicocelectomy. Red nodes indicate proteins exclusively or overexpressed in the pre-varicocelectomy group, green nodes indicate proteins exclusively expressed in the post-varicocelectomy group and yellow nodes indicate proteins not differentially expressed between both groups. Interactions were inferred using the BisoGenet plugin in the Cytoscape 2.8.2 software.

the balance between synthesis and degradation of proteins in cells (Shi *et al*., 1998). A study with boar semen found that HSP90 is associated with the reduction of sperm motility (Huang *et al*., 2000), although it is also associated with protecting cells against oxidative damage (Conconi *et al*., 1996).

The main function overrepresented in the pre-varicocelectomy group is the TPR-binding. TPR-binding domains are involved in protein–protein interactions (Cortajarena *et al*., 2004) and form macrocomplexes (Smith *et al*., 1993). TPR domains are important for the functioning of certain chaperones in the cell cycle, transcription and protein transport (Blatch and Lässle, 1999). The molecular chaperones ‘heat-shock protein 90A’ and ‘heat-shock protein 90B’ have TPR-binding domains, which explain the overrepresentation of this function in pre-varicocelectomy group.

The function of biosynthesis and metabolism of NO is the most abundant function overrepresented in the functional enrichment analysis in the pre-varicocelectomy group (Fig. 2). Varicocele is associated with elevated reactive oxygen species (ROS) and decrease of antioxidant activity (Hendin *et al*., 1999), as well as increased NO production. Ozbek *et al*. (2000) found levels of NO in the internal spermatic veins of infertile patients to be twice that in controls, and proposed that this increase in patients with varicocele may be

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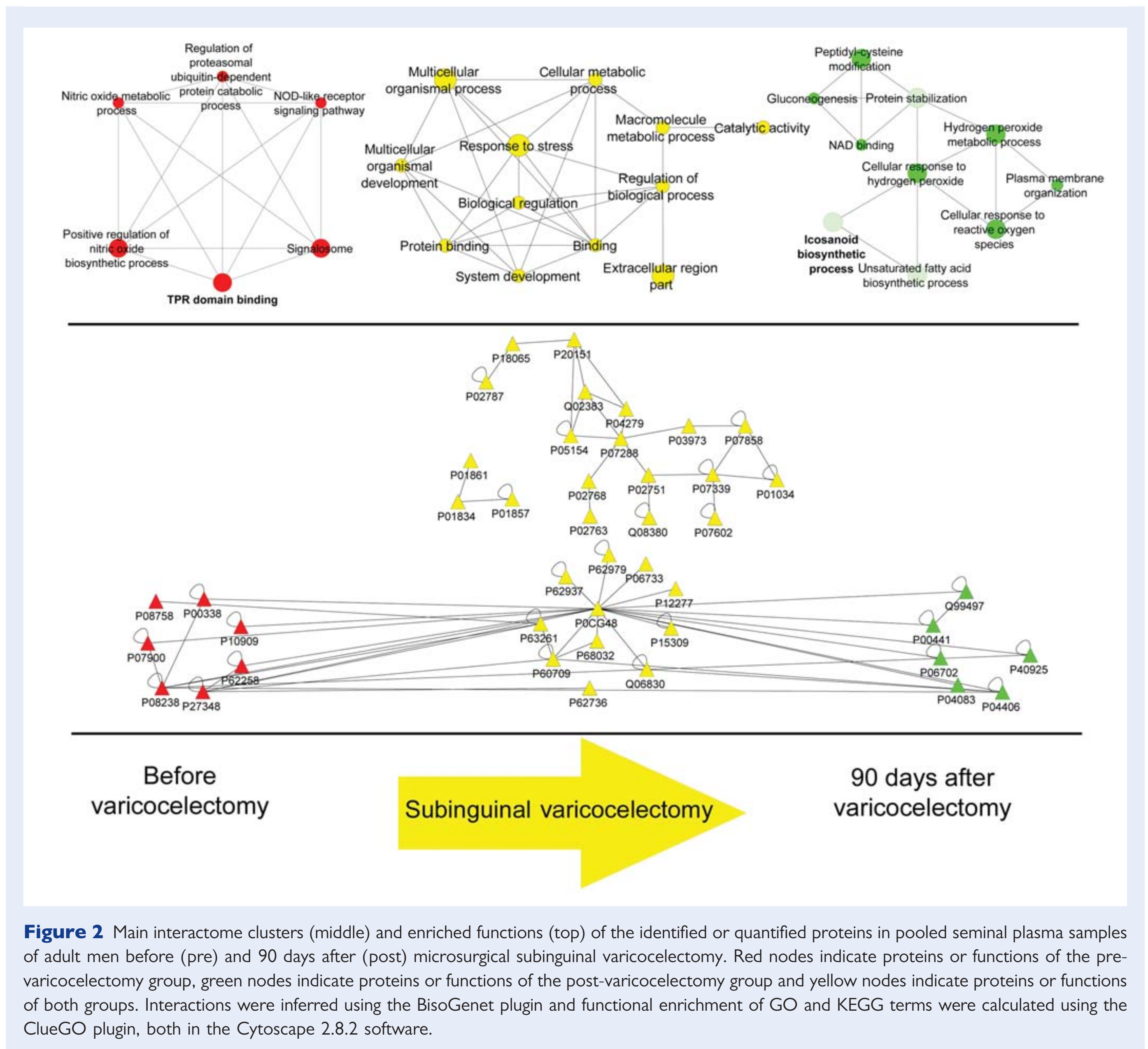

Figure 2 Main interactome clusters (middle) and enriched functions (top) of the identified or quantified proteins in pooled seminal plasma samples of adult men before (pre) and 90 days after (post) microsurgical subinguinal varicocelectomy. Red nodes indicate proteins or functions of the pre-varicocelectomy group, green nodes indicate proteins or functions of the post-varicocelectomy group and yellow nodes indicate proteins or functions of both groups. Interactions were inferred using the BisoGenet plugin and functional enrichment of GO and KEGG terms were calculated using the ClueGO plugin, both in the Cytoscape 2.8.2 software.

responsible for sperm dysfunction and its association with infertility (Ozbek *et al*., 2000). Different studies have reported high levels of seminal oxidative stress in men with varicocele, suggesting that sperm dysfunction is a consequence of oxidative stress (Zini *et al*., 2000; Meucci *et al*., 2003; Pasqualotto *et al*., 2008). De Lamirande and Gagnon (1992) showed that low sperm motility was associated with high levels of oxidative stress, because high concentrations of ROS inhibit enzymes related to oxidative phosphorilation and/or glycolysis, thereby limiting the production of ATP (de Lamirande and Gagnon, 1992).

Post-varicocelectomy proteins

In the post-varicocelectomy group, the proteins DJ-1 (Q99497), superoxide dismutase (SOD) [Cu-Zn] (P0441), S100-A9 (P06702), annexin 1 (P04083), glyceraldehyde 3-phosphate dehydrogenase (G3P_HUMAN) and malate dehydrogenase (MDH) (P40925) relate to each other and with conserved proteins. These proteins have over-represented functions such as cellular response to reactive oxygen species, gluconeogenesis, NAD-binding and protein stabilization (Fig. 2).

DJ-1 and SOD are linked to response to ROS (Fig. 2). SOD protein has a central role because it reduces superoxide anions (Fridovich, 1995). A study performed by Ishii *et al*. (2005) showed that ROS are cytotoxic mediators in spermatogenic cells during heat stress possibly oxidizing important molecules such as polyunsaturated fatty acids (Peltola *et al*., 1995; Ishii *et al*., 2005), resulting in the death of these cells.

DJ-1 has previously been described in sperm and in seminal plasma. Yoshida *et al*. (2003) found DJ-1 levels to be decreased in infertile men. Immunohistochemistry showed this protein to be present in

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the back of the head and in front of the midpiece, and in the sperm flagellum, suggesting their role in oocyte penetration and flagellar movement and that their presence is related to fertility (Yoshida *et al*., 2003). These functions were also found in experiments with rats and mice (Klinefelter *et al*., 2002; Okada *et al*., 2002). It should be noted that ROS are important mediators of sperm capacitation (Aitken *et al*., 1995; de Lamirande and Gagnon, 1995), and enriched cellular response to ROS indicates that sperm are possibly reacting to ROS in a more physiological manner.

Annexin 1, also known as lipocortin, has a strong anti-inflammatory activity of inhibiting phospholipase A2 (Davidson *et al*., 1987). The S100A9 protein is a member of a family of proteins called S100. The S100 proteins are localized in the cytoplasm and/or nucleus of a wide variety of cells, and are involved in regulating a number of cellular processes such as cell cycle progression and cell differentiation (Bjork *et al*., 2009). Their presence in seminal plasma may be due to a residual spermatogenic effluate from the seminiferous tubule luminal fluid, indicated improved spermatogenic conditions.

Glyceraldehyde 3-phosphate dehydrogenase (G3P) and MDH have a role in gluconeogenesis and NAD-binding. These two proteins are from a family of nucleotide-binding proteins, and belong to the group of NAD-dependent dehydrogenases (Minárik *et al*., 2002). There are no reports in the literature about gluconeogenesis in human spermatozoa, and Marin *et al*. (2003) demonstrated the absence of gluconeogenesis in boar spermatozoa. However, they concluded that the glycolytic metabolism may be different among species (Marin *et al*., 2003). The NAD-binding function in present both in gluconeogenesis (Marin *et al*., 2003) as in glycolysis, the main source of energy of human sperm (Williams and Ford, 2001). Glycolysis plays an important role in the generation of ATP to support sperm motility (Ford, 2006). To support this idea, Miki *et al*. (2004) found that knockout mice for G3P gene had immotile sperm (Miki *et al*., 2004). Thus, the absence of these two proteins in the semen of pre-varicocelectomy patients, confirms some reduced seminal parameters, and it is possible to suggest G3P protein as a biomarker of mobile sperm.

In conclusion, the proteins found in both groups possess functions usually found in human semen. While many of these functions are conserved, we observed a shift in state from a responsive-to-stress condition before varicocele correction to a responsive-to-environment condition after varicocelectomy. This shift back to homeostasis observed in the post-genomic pathways of seminal plasma suggests that varicocele correction is beneficial in returning semen to a physiological state.

Authors' roles

M.C.: conception and design of the study, acquisition of samples for analysis, interpretation of data, drafting of the article. Final approval for submission; P.I.L.: acquisition of samples for analysis, interpretation of data, revision of the article; P.T.D.: acquisition of samples for analysis, interpretation of data, revision of the article; V.M.C.: mass spectrometry - sample acquisition, analysis and discussion of results - final approval for submission; K.H.M.C.: mass spectrometry – sample acquisition, analysis of results, revision of the article. Final approval for submission; C.A.: interpretation of data, discussion of results, revision of the article; R.F.: patient screening, clinical evaluation, discussion of results, revision of the article; R.P.B.: conception and design of the study, acquisition of samples for analysis, interpretation of data, drafting of the article.

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Conflict of interest

None declared.

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